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Validation of the 4B5 rabbit monoclonal antibody in determining Her2/neu status in breast cancer

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HER2 overexpression in breast cancer is associated with worse clinical outcome. To select patients for anti-Her2-based therapy immunohistochemistry is commonly performed as a first step to assess Her2 status. However, interobserver and interlaboratory variability can significantly compromise adequate assessment of Her2 status. In addition, immunohistochemistry does not always result in an unambiguous test result requiring additional testing for Her2 gene amplification. This study aimed to improve the reliability of Her2 immunohistochemistry by using rabbit monoclonal antibody 4B5 as an alternative to mouse monoclonal antibody CB11 routinely used in our laboratory. Therefore, 283 breast adenocarcinomas were included in a tissue microarray. Immunohistochemistry using the 4B5 and CB11 antibodies, and fluorescence and chromogenic *in situ* hybridization (FISH or CISH) were performed. Immunohistochemistry was scored by two independent investigators. We found that 4B5 staining was more distinct than CB11 staining. For CB11 staining, there were 12% (BV) and 5% (JW) 2+ scores compared with 4% (BV) and 2% (JW) for 4B5. There was a strong trend towards higher interobserver agreement for 4B5 compared with CB11 (4B5: κ 0.87, 95% CI 0.79–0.96; CB11: κ 0.77, 95% CI 0.66–0.88). There were no significant differences in sensitivity, specificity and predictive values between CB11 and 4B5. Our results indicate that the 4B5 antibody provides more robust assessment of immunohistochemical Her2/neu status and will reduce the number of gene amplification tests compared with CB11. However, for tumours with a 2+ score additional gene amplification measurement using FISH or CISH remains necessary.
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Keywords: breast carcinoma; immunohistochemistry; validation study; Her2/neu; rabbit monoclonal antibody

The status of the Her2 receptor is an important factor in the prognosis and treatment choice in primary breast carcinoma.[1] Mortality and recurrence are reduced in patients with Her2/neu-positive breast carcinoma that receive adjuvant therapy with both the humanized anti-Her2/neu monoclonal antibody therapy trastuzumab and chemotherapy.[2–5]

Trastuzumab therapy, however is associated with cardiotoxicity, in 2–4.7% of patients when used as monotherapy, but in up to 27% when given concomitantly with anthracycline and cyclophosfamide therapy.[6] Therefore, this treatment is only given to patients with confirmed Her2/neu-positive breast carcinoma and adequate left ventricle ejection fraction. Several methods are used to assess Her2/neu status in breast cancer. Measurement of gene amplification using fluorescence *in situ* hybridization (FISH) or chromogenic *in situ* hybridization (CISH) is considered to be the 'gold standard' in the assessment of Her2/neu status.[7–9] However, the most commonly used first-line method to determine Her2/neu status is immunohistochemistry. Immunohistochemistry is relatively inexpensive and a routinely used technique in pathology laboratories, which makes it easy to implement. Immunohistochemistry results in a Her2/neu score ranging from 0 (no expression) to 3+ (strong complete tumour cell membrane expression).[10] This semiquantitative scoring system does not always result in a clear

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positive or negative Her2/neu amplification status. It is generally agreed that when a Her2 score is ambiguous (2+ score) a gene amplification measurement has to be performed.[7,11,12] Another setback of Her2 immunohistochemistry is the significant interobserver variation and poor interlaboratory reproducibility.[13–16] As accurate diagnostic assessment of HER2/neu is essential for the appropriate application of trastuzumab-containing treatment regiments, the concordance between immunohistochemistry and the 'gold standard' gene amplification assessment needs to be as high as possible. Therefore, immunohistochemistry staining should be improved to optimize accurate estimation of the HER2/neu status.

In recent years, rabbit monoclonal antibodies have been developed, which show higher affinity and specificity than mouse monoclonal or rabbit polyclonal antibodies resulting in more reliable staining results.[17,18] To improve Her2 immunohistochemistry reliability in our laboratory, we tested the potential of the rabbit monoclonal antibody 4B5 directed against Her2/neu as an alternative to the mouse monoclonal CB11 directed against Her2/neu, using both a CISH array for Her2/neu and a Her2/neu FISH array as reference.

Materials and methods

Patients

To determine the size of the series, a power analysis was performed. In this power analysis, we considered HER2/neu immunohistochemistry negative if the staining pattern and intensity is equivalent to score '0' or '1+' and positive if it is equivalent to score '3+'. 2+ cases are not taken into account in this power analysis, because they are clinically uninformative. We assumed 10% 2+ cases. We also assumed that the rabbit monoclonal antibody has a better sensitivity and specificity than the mouse monoclonal antibody CB11. Furthermore, a loss of 20% cases because of unavailability of tumour material or uninterpretability of one or more of the stainings was assumed. On the basis of these assumptions, a sample size of 280 was required to achieve 90% power to detect an odds ratio of 3.000 using a two-sided McNemar test with a significance level of 0.05000. The odds ratio is equivalent to a difference between two paired proportions of 0.100, which occurs when the proportion T_N negative *vs* T_O positive is 0.150 and the proportion T_N positive *vs* T_O negative is 0.050. A total of 283 consecutive female patients treated for a primary operable invasive carcinoma of the breast at the University Medical Center Groningen between January 2002 and December 2005 were included in this study. Afterwards eight patients were excluded because no representative material was available in the tissue microarray blocks. The analyses have been performed on the resulting group of 275 patients.

Table 1 Patient and tumour characteristics

	n (%)
Age at diagnosis	
Median (range)	60 (29–90)
Menopausal status	
Premenopausal	78 (28)
Postmenopausal	197 (72)
Pathological tumour size (mm)	
Median (range)	22 (1–140)
Pathological tumour type	
IDC	109 (40)
IDC and DCIS	110 (40)
ILC	25 (9)
ILC and LCIS	6 (2)
other	25 (9)
Grade of differentiation (invasive)	
Well	82 (30)
Moderate	114 (41)
Poor	79 (29)

n, number of cases; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma *in situ*; other, other specific subtypes of invasive breast carcinoma.

Patient and tumour characteristics were obtained retrospectively from hospital records and are summarized in Table 1.

Tissue Microarray Construction

From the patient's tumour paraffin block, three 0.6 mm core samples of the most representative tumour area were included in a tissue microarray. The technique of tissue microarray production has been described and validated for breast carcinoma by others.[19,20] In the latter study, authors showed that the concordance between the tissue microarray with the whole tissue sections was over 97% if three 0.6 mm core samples per tumour were included in the TMA. In brief, tissue microarrays were compiled as follows: the most representative tumour area was marked on the original haematoxylin and eosin (H&E)-stained section. Using this section as an orientation, three 0.6 mm core punches were taken from the selected area in the donor blocks and mounted in a recipient block, using a manual tissue microarray device (Beecher Instruments, Silver Springs, MD, USA). In total, seven tissue microarray blocks, each containing tumour cores from 40 to 50 patients, were made. Using a standard microtome, 3 μm sections were cut from these tissue microarray blocks.

Immunohistochemistry, FISH and CISH

Immunohistochemistry for rabbit monoclonal antibody 4B5 (PATHWAY® HER-2/neu (4B5) rabbit

monoclonal antibody, Ventana Medical Systems, Illkirch, Cedex, France) and mouse monoclonal antibody CB11 (PATHWAY HER-2/neu (CB11) mouse monoclonal antibody, Ventana Medical Systems) was performed on the tissue microarray sections using the automated Benchmark® platform (Ventana Medical Systems) and according to the manufacturers recommendations. FISH (PathVysion HER-2 DNA Probe Kit, Vysis Inc., Downers Grove, IL, USA) and CISH (SPoT-Light® HER2 CISH™ Kit, Zymed, Carlsbad, CA, USA) assays were performed according the manufacturers recommendations.

Evaluation of Immunohistochemistry, FISH and CISH

Scoring of the CB11 and 4B5 immunohistochemistry stainings was performed independently by an experienced pathologist (JW) and a senior resident (BV). Her-2/neu expression was graded as recommended by the HercepTest™ scoring guidelines: 0: no staining at all or membrane staining in $<10\%$ of the tumour cells; 1+: a faint/barely perceptible partial membrane staining in $>10\%$ of the tumour cells; 2+: weak-to-moderate complete membrane staining in $>10\%$ of the tumour cells; 3+: strong complete membrane staining in $>10\%$.

Fluorescence *in situ* hybridization was scored according to the ASCO guidelines: the ratio of 20 cells was calculated. A ratio <1.8 was considered negative, a ratio >2.2 was considered positive. For the equivocal cases another 20 cells were counted. In these cases, a ratio 2 was considered positive. A ratio <2 was considered negative. For CISH, tumours with at least five signals in more than 50% of the tumour cells were considered positive.

Data Analysis

All statistical analyses were performed using the SPSS 14.0 software package.

The feasibility of both staining methods was compared. The number of informative test results and the number of 2+ scores were calculated for each of the staining methods. Agreement between immunohistochemistry results and *in situ* hybridization results were calculated in a cross tabulation using a Pearson χ^2-test. Sensitivity and specificity were calculated using two methods, including and excluding 2+ scores. In the first method, 2+ scores were considered a positive test result. This method is most commonly used in literature and was performed to compare our results to those found in other studies. 2+ scores are, however, clinically uninformative and were therefore eliminated from analysis in the second method. The positive predictive value (PPV) was calculated by dividing the number of cases in the immunohistochemistry 3+ group with amplification on FISH or CISH by the total number of cases with an immunohistochemistry 3+ score. The negative predictive value (NPV) was calculated by dividing the number of cases immunohistochemistry score 0 or 1+ without amplification on FISH or CISH by the total number of patients with an immunohistochemistry 0 or 1+ score. For sensitivity, specificity and predictive values a 95% CI interval was calculated. Interobserver agreement was calculated in a cross tabulation using Cohen's κ-test.

Results

Feasibility

In Figure 1, an example of both a 4B5 and a CB11 staining for 1 case is shown. In general, the 4B5 staining was more distinct compared with the CB11 staining. Also the 4B5 staining showed less nonspecific cytoplasmic staining.

Test Characteristics

Of the 275 cases included, 262 (95%) of the cases were adequately represented in the tissue micro-

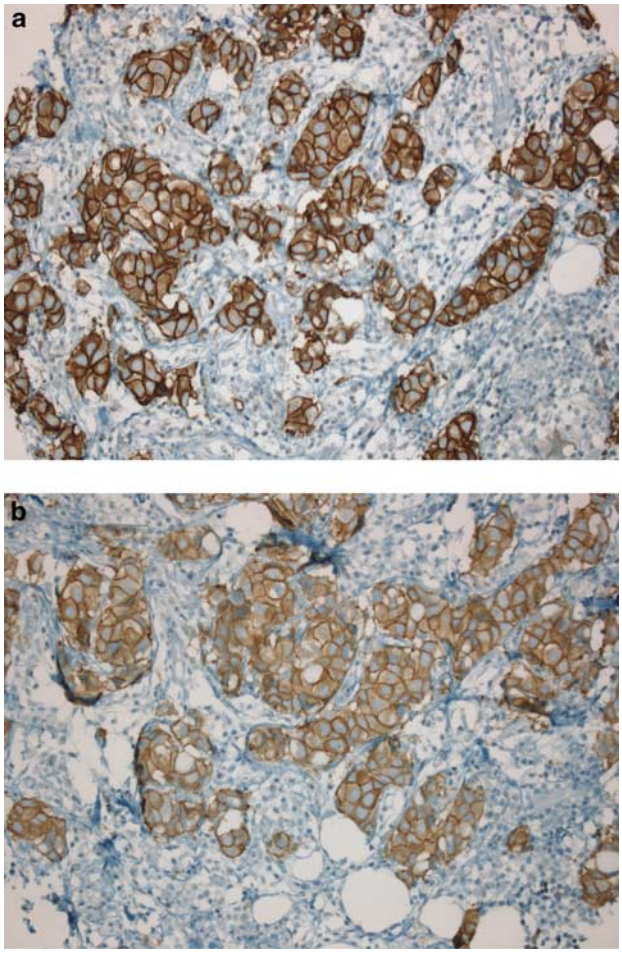

Figure 1 Comparison of 3+ case for 4B5 and CB11. (**a**) 4B5. (**b**) CB11.

Table 2 Correlation of 4B5 and CB11 for observer BV (a) and JW (b)

(a)

	CB11 BV				
4B5 BV	*0*	*1*	*2*	*3*	*Total*
0	149 (95)	20 (50)	4 (14)	0 (0)	173 (71)
1	7 (4)	19 (48)	14 (50)	0 (0)	40 (16)
2	1 (1)	1 (2)	8 (29)	0 (0)	13 (4)
3	0 (0)	0 (0)	2 (7)	19 (100)	21(9)
Total	157 (100)	40 (100)	28 (100)	19 (100)	244 (100)
χ^2: $P \leq 0.001$					

(b)

	CB11 JW				
4B5 JW	*0*	*1*	*2*	*3*	*Total*
0	106 (96)	28 (35)	0 (0)	0 (0)	134 (60)
1	5 (4)	51 (64)	6 (50)	0 (0)	62 (28)
2	0 (0)	1 (1)	4 (33)	0 (0)	5 (2)
3	0 (0)	0 (0)	2 (17)	20 (100)	22 (10)
Total	111 (100)	80 (100)	12 (100)	20 (100)	223 (100)
χ^2: $P \leq 0.001$					

CB11: mouse monoclonal antibody CB11; 4B5: rabbit monoclonal antibody 4B5; BvdV: Bert van der Vegt; JW: Jelle Wesseling.

array. Immunohistochemistry could be evaluated in 83 (JW) and 90% (BV) of the cases for 4B5, in 87 (JW) and 91% (BV) of the cases for CB11. Differences in evaluation percentages between authors have been caused by disagreement on the overall percentage of tumour available in the core punches and the relative percentages of invasive tumour *vs in situ* tumour available in the core punches. FISH could be evaluated in 90% and CISH in 92% of the cases.

Agreement

In 230 of the 240 assessable cases (96%), there was agreement between FISH and CISH scoring (κ 0.837, 95% CI 0.737–0.937). There was an agreement of 80 (BV) and 81% (JW) between the scoring results of 4B5 and CB11 (Table 2). The disagreement between scores is mainly caused by the 2+ scores. There was a reduction of more than 50% for the number of cases scored as 2+ when comparing CB11 with 4B5, respectively, 28 cases (12%) *vs* 13 cases (4%) (BV) and 12 cases (5%) *vs* 5 cases (2%) (JW). When excluding the 2+ cases from analysis, there was no difference between both antibodies for the classification of cases as amplified or not amplified (McNemar's test $P = 1.0$, data not shown) eg, there would be no clinical consequences when using either of the antibodies. In Tables 3 and 4, the results for the concordance between immunohistochemistry and respectively FISH and CISH are shown. Sensitivity (including and excluding 2+ scores), specificity (including and excluding 2+ scores), PPV and NPV of 4B5 and CB11 using

Table 3 comparison of immunohistochemistry with FISH

	Amplification	*No amplification*	*Total*
IHC CB11 BvdV			
0	3 (8)	150 (74)	153 (64)
1	2 (5)	38 (19)	40 (16)
2	13 (35)	15 (7)	28 (12)
3	19 (52)	0 (0)	19 (8)
Total	37 (100)	203 (100)	240 (100)
χ^2: $P \leq 0.001$			
IHC 4B5 BvdV			
0	5 (14)	165 (81)	170 (71)
1	4 (11)	36 (18)	40 (17)
2	7 (19)	3 (1)	10 (4)
3	21 (56)	0 (0)	21 (8)
Total	37 (100)	204 (100)	241 (100)
χ^2: $P \leq 0.001$			
IHC CB11 JW			
0	2 (5)	116 (59)	118 (51)
1	5 (14)	78 (40)	83 (36)
2	10 (27)	2 (1)	12 (5)
3	20 (54)	0 (0)	20 (8)
Total	37 (100)	196 (100)	233 (100)
χ^2: $P \leq 0.001$			
IHC 4B5 JW			
0	3 (8)	131 (69)	134 (60)
1	7 (20)	57 (30)	64 (28)
2	4 (11)	1 (1)	5 (2)
3	22 (61)	0 (0)	22 (10)
Total	36 (100)	189 (100)	225 (100)
χ^2: $P \leq 0.001$			

IHC: immunohistochemistry; CB11: mouse monoclonal antibody CB11; 4B5: rabbit monoclonal antibody 4b5; BvdV: Bert van der Vegt; JW: Jelle Wesseling.

both FISH and CISH as reference are shown in Table 5. Sensitivity, specificity, PPV and NPV did not show any significant differences between both antibodies and between both observers. However, there was a near significant difference in specificity (including 2+ scores) for the lesser experienced observer (BV) between 4B5 and CB11: (4B5: 0.99, 95% CI 0.95–1.0; CB11: 0.93, 95% CI 0.88–0.96). Although not significant, there was a trend towards an increased concordance between observers for 4B5 compared with CB11 (4B5: κ 0.87, 95% CI 0.79–0.96; CB11: κ 0.77, 95% CI 0.66–0.88) (Table 6).

Table 4 comparison of immunohistochemistry with CISH

	Amplification	*No amplification*	*Total*
IHC CB11 BvdV			
0	2 (6)	156 (76)	158 (65)
1	4 (11)	35 (17)	39 (16)
2	11 (31)	15 (7)	26 (11)
3	19 (52)	0 (0)	19 (8)
Total	36 (100)	206 (100)	242 (100)
χ^2: $P \leq 0.001$			
IHC 4B5 BvdV			
0	3 (8)	171 (83)	174 (71)
1	6 (17)	33 (16)	39 (16)
2	6 (17)	3 (1)	9 (4)
3	21 (58)	0 (0)	21 (9)
Total	36 (100)	207 (100)	243 (100)
χ^2: $P \leq 0.001$			
IHC CB11 JW			
0	1 (3)	121 (61)	122 (52)
1	6 (17)	74 (37)	80 (34)
2	9 (25)	3 (2)	12 (5)
3	20 (55)	0 (0)	20 (9)
Total	36 (100)	198 (100)	234 (100)
χ^2: $P \leq 0.001$			
IHC 4B5 JW			
0	2 (6)	134 (71)	136 (60)
1	9 (25)	53 (28)	62 (28)
2	3 (8)	2 (1)	5 (2)
3	22 (61)	0 (0)	22 (10)
Total	36 (100)	189 (100)	225 (100)
χ^2: $P \leq 0.001$			

4B5, rabbit monoclonal antibody 4B5; BvdV, Bert van der Vegt; CB11, mouse monoclonal antibody CB11; IHC, immunohistochemistry; JW, Jelle Wesseling.

Discussion

In this study, we tested the potential of the 4B5 anti-Her2 rabbit monoclonal antibody by comparing it with the CB11 anti-Her2 mouse monoclonal antibody on a consecutive series of invasive breast adenocarcinomas, using both FISH and CISH as reference. We found that 4B5 staining was more distinct and showed less nonspecific cytoplasmic background staining, which led to a more than 50% reduction of the number of 2+ scores for 4B5 compared with CB11. In this well powered study we found no significant differences in sensitivity, specificity, predictive values and interobserver concordance between 4B5 and CB11. However, there was a trend towards a higher specificity for 4B5 for one of the observers (BV) and a trend towards an increased interobserver concordance for 4B5. The – nonsignificant – differences in the test results of

Table 6 Concordance between observers

	κ	*95% CI*
4B5	0.87	0.79–0.96
CB11	0.77	0.66–0.88

Table 5 sensitivity, specificity and predictive values for immunohistochemistry compared with FISH and CISH

	FISH						
	Sensitivity	*Specificity*	*Sensitivity (excl 2+ scores)*	*Specificity (excl 2+ scores)*	*PPV*	*NPV*	*% 2+*
4B5 BvdV	0.75 (0.57–0.87)	0.99 (0.95–1.0)	0.70 (0.50–0.85)	1.0 (0.98–1.0)	1.0 (0.81–1.0)	0.96 (0.92–0.98)	4
CB11 BvdV	0.83 (0.67–0.93)	0.93 (0.88–0.96)	0.79 (0.57–0.92)	1.0 (0.98–1.0)	1.0 (0.79–1.0)	0.97 (0.94–0.99)	12
4B5 JW	0.69 (0.52–0.83)	0.99 (0.96–1.0)	0.69 (0.50–0.83)	1.0 (0.98–1.0)	1.0 (0.82–1.0)	0.95 (0.91–0.97)	2
CB11 JW	0.81 (0.63–0.91)	0.99 (0.96–1.0)	0.74 (0.53–0.88)	1.0 (0.98–1.0)	1.0 (0.80–1.0)	0.97 (0.93–0.98)	5
	CISH						
	Sensitivity	*Specificity*	*Sensitivity (excl 2+ scores)*	*Specificity (excl 2+ scores)*	*PPV*	*NPV*	*% 2+*
4B5 BvdV	0.76 (0.58–0.88)	0.99 (0.95–1.0)	0.70 (0.50–0.85)	1.0 (0.98–1.0)	1.0 (0.81–1.0)	0.96 (0.92–0.98)	4
CB11 BvdV	0.86 (0.70–0.95)	0.93 (0.88–0.96)	0.76 (0.54–0.90)	1.0 (0.98–1.0)	1.0 (0.80–1.0)	0.97 (0.93–0.99)	11
4B5 JW	0.72 (0.55–0.85)	0.99 (0.96–1.0)	0.67 (0.48–0.81)	1.0 (0.97–1.0)	1.0 (0.82–1.0)	0.94 (0.90–0.97)	2
CB11 JW	0.81 (0.64–0.91)	0.99 (0.96–1.0)	0.74 (0.53–0.88)	1.0 (0.98–1.0)	1.0 (0.80–1.0)	0.97 (0.93–0.98)	5

4B5, rabbit monoclonal antibody 4b5; BvdV, Bert van der Vegt; CB11, mouse monoclonal antibody CB11; IHC, immunohistochemistry; JW, Jelle Wesseling; NPV, negative predictive value; PPV, positive predictive value.

both observers would have no consequences for clinical decision making regarding the Her2 status of the carcinomas investigated.

Gene amplification measurement is considered to be the 'gold standard' for Her2/neu status assessment,[7–9] although it suffers from variability between institutes[21,22,] and the sensitivity varies between detection methods used.[9] Traditionally, gene amplification measurement has been performed using FISH assays for Her2/neu gene amplification. This method, however, has several disadvantages: FISH is expensive, is time demanding,[8] is not readily accommodated in most pathology laboratories and is accompanied by technical challenges.[23] More recently CISH assays have been developed. Instead of the fluorogens used in FISH, this technique uses chromogens. This has several advantages[24]: where FISH requires a fluorescence microscope for interpretation, CISH can be interpreted using a normal bright field microscope. CISH allows analysis of tumour morphology, making it possible to interpret tumour heterogeneity and gene copy number in different components of the tumour (an invasive and an *in situ* component). Also FISH signals are labile and fade over time, but CISH produces a permanent staining.

Many authors have compared CISH with FISH (reviewed by Lambros *et al*[24]). Most studies find an agreement of both methods of more than 90%. In a multicenter study, where pathology laboratories blindly performed CISH on cases from each other this method was validated.[25] That study also reported an intra- and interobserver agreement of over 90% and concluded that CISH is a viable alternative to FISH. In this study, the agreement between FISH and CISH was 96%, which is in line with the conclusions of those earlier studies. Of the 10 discordant cases in our series 5 cases contained an *in situ* component. This tumour heterogeneity might have led to the discordance because distinction between the invasive and the *in situ* component can be difficult in FISH where tumour morphology is not readily recognized in all cases.

Most pathology laboratories use immunohistochemistry to assess Her2/neu status. Immunohistochemistry is not only less expensive than *in situ* methods, there is also much experience with immunohistochemistry, which makes it a method that is easily implemented in the daily practice of pathology laboratories. Immunohistochemistry, however, has several disadvantages: immunohistochemistry is scored semiquantitively, which leads to a $0-3+$ score rather than to a clear amplification/no amplification outcome. Scores 0 and $1+$ are considered non-amplified and score $3+$ is considered amplified. An ideal Her2 antibody has a low number of indeterminate $(2+)$ cases and a high PPV and NPV. We found that 4B5 staining compared with CB11 was more distinct and showed less nonspecific cytoplasmic background staining. This has led to an increase in interobserver concordance (4B5: κ 0.87, 95% CI 0.79–0.96; CB11: κ 0.77, 95% CI 0.66–0.88) that showed a trend towards significance. Interobserver concordance is essential because it has far reaching consequences for the choice of therapy regimen and response to therapy. A few studies have assessed interobserver concordance for CB11. Two of those studies found an interobserver concordance of 0.74, which is comparable with our results (0.77).[13,15] In a study by Tsuda *et al*[16] an interobserver concordance of only 0.29 was found for CB11. However, this study used a different method to calculate concordance. The concordance of 0.87 that we found for 4B5 is considered almost perfect,[26] which underlines that 4B5 is a safe method to assess Her2 status in breast cancer. To our knowledge, no other studies have assessed interobserver concordance for 4B5. Interlaboratory concordance was not assessed in this study. One study mentions a perfect interlaboratory concordance on a very limited number of cases.[27] Future studies are needed to assess this issue.

In this series, a cutoff of 10% staining was used for scoring Her2 immunohistochemistry. This cutoff was chosen based on Dutch guidelines, which differ from the ASCO guideline using a cutoff of 30%.

Table 7 Sensitivity, specificity, predictive and concordance for CB11 from literature

Author	*Study size*	*Sensitivity*	*Specificity*	*Method*	*PPV*	*NPV*
Kakar *et al* [30]	112	0.93 (0.66–1.0)[a]	0.98 (0.90–1.0)[a]	2	0.88 (0.60–0.98)[a]	0.99 (0.92–1.0)[a]
Bartlett *et al*[13]	213	0.85 (0.70–0.94)[a]	0.69 (0.62–0.76)[a]	1	0.40 (0.30–0.51)[a]	0.95 (0.89–0.98)[a]
Press *et al*[9]	74	0.721 (0.56–0.85)	1.00 (0.95–1.0)	1		
Press *et al*[21]	64	0.95 (0.74–1.0)	0.84 (0.70–0.93)	1		
Ricardo *et al*[31]	190	0.52 (0.37–0.67)[a]	0.98 (0.93–1.0)[a]	2	0.92 (0.72–0.99)[a]	0.83 (0.75–0.89)[a]
Powell *et al*[27]	178[b]	0.92 (0.83–0.96)[a]	0.91 (0.82–0.96)[a]	1	0.92 (0.83–0.96)[a]	0.91 (0.82–0.96)[a]
	144[b]	0.74 (0.64–0.83)[a]	0.91 (0.80–0.97)[a]	1	0.93 (0.83–0.97)[a]	0.71 (0.59–0.80)[a]
Egervari *et al*[29]	199	0.83 (0.58–0.96)[a]	0.99 (0.96–1.0)[a]	2	0.94 (0.68–1.0)[a]	0.98 (0.94–0.99)[a]

Method 1, 2+ and 3+ scores considered positive; Method 2, 2+ cases excluded from analysis; PPV, positive predictive value; NPV, negative predictive value.

[a]Calculated from the data available in the article.

[b]Two separate subsets were studied in the article.

However, this difference in cutoff did not cause a significant change in the results.

The number of cases that were scored as 2+ was lower for 4B5. For 4B5, observers scored 4 and 2% 2+ cases; for CB11 this was 12 and 5%. The number of CB11 2+ cases in this study is comparable the with numbers in literature, where the number of cases scored 2+ using an mouse monoclonal antibody ranges from 2 to 20.5%, and is usually around 10%.[28] We expect that the introduction of 4B5 will reduce the number of FISH or CISH assays that will have to be performed, which leads to a reduction in costs. In this well powered study, this decrease of indeterminate cases did not lead to a significant difference in sensitivity, specificity, or predictive power between CB11 and 4B5. Small numbers did not allow a statistically reliable subgroup analysis to assess differences in sensitivity, specificity and predictive values for the immunohistochemistry 2+ cases of both antibodies using FISH or CISH as a reference. In table 7, the results from other studies assessing sensitivity, specificity and predictive values of CB11 are shown. Most studies considered 2+ cases to be amplified for the calculations. As discussed before 2+ cases correlate with gene amplification very poorly.[7,11,12] We believe that 2+ cases should be excluded from analysis when assessing sensitivity, specificity and predictive values. The values for sensitivity, specificity and predictive values that we found in this study are comparable with those found in literature. Powell *et al*[27] performed a study comparing 4B5 with CB11 using FISH as a reference in two subsets of cases, one containing samples from a single institution, and one containing samples from a multicenter tissue bank. In that study the levels of sensitivity were higher and the levels of specificity were lower than in this study. However, these differences were not significant for the subset best comparable with this study group, ie, the single institution subset. PPV and NPV show no significant difference between both antibodies, which underlines that the lower number of indeterminate cases which led to the increase in sensitivity and specificity did not cause a loss of predictive value of the test. In a group of 199 invasive breast cancers, Egervari *et al*[29] compared the 4B5 antibody with a number of antibodies including the CB11 antibody using FISH as a reference. When recalculating their results using the method described earlier they had sensitivity (0.76 (0.5–0.92)), specificity (0.99 (0.96–1.0)), PPV (0.93 (0.64–1.0)) and NPV (0.97 (0.93-0.99)) that does not differ significantly to our results. Although they suggest a lower sensitivity of 4B5 compared with CB11, this result is not significant (Table 7).

In conclusion, we showed that the novel 4B5 rabbit monoclonal anti-Her2/neu antibody has a good agreement with both FISH and CISH and has a sensitivity, a specificity and predictive values comparable with CB11. We found a reduction of more than 50% in the number of indeterminate cases for 4B5 and an increase in interobserver concordance. Our results indicate that immunohistochemistry using the 4B5 antibody provides more robust and hence more reliable assessment of the Her2/neu status detection and will reduce the number of gene amplification tests compared with the CB11 antibody. However, for patients with a 2+ score additional gene amplification measurement using FISH or CISH remains mandatory.

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Disclosure/conflict of interest

None.

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